

BIOCHEMICAL ASSAYS ON PLASMINOGEN ACTIVATORS
AND
HORMONES FROM KIDNEY SOURCES

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Ever since Williams (1) first described the plasminogen activator from human urine and Sobel (2) named it urokinase (UK) and the vascular activator was described by Aoki (3) the activator system in man has become more complex. What at first appeared to be single well defined proteins have now been shown to exist in multiple molecular forms. UK can exist as a single or two chain molecule (4) and as a fully active degraded two chain form (5), or a degraded single chain form (6). Vascular activator, which is now more widely known as tissue plasminogen activator (t-PA), can also exist as a single or two chain molecule (7) and has been reported to also have a degraded form (8). In any normal system the presence of many different molecular forms is highly possible and based on the potential for different responses in different assays can lead to a very complex problem with interpretation (9).

Determination of activity levels can be further complicated by inhibitors to the activator as well as to plasmin, the end product of the enzymatic activity of the activators. The use of amidolytic and esterolytic

assays that measure the enzyme activity directly can give a very different response than those assays which are of the indirect nature requiring the measurement of the enzyme plasmin, the results of the activation of plasminogen by the activator.

These investigations were established for the purpose of analyzing the conditioned media from human embryonic kidney cell subpopulations separated in space by electrophoresis. This data is based on the experiments performed on STS-8 on the Continuous Flow Electrophoresis System (CFES), developed by McDonnell-Douglas Astronautics Co. The primary biological activity that was analyzed was plasminogen activator activity, but some assays for erythropoietin and human granulocyte colony stimulating activity were also performed.

The cell preparation prior to separation and the subsequent culturing of the sub-fractions were not part of these investigations and were accomplished at the Johnson Space Center.

ASSAYS

FIBRIN PLATE LYSIS ASSAY (FPL): The standard assay described by Brakkman (10) and modified by the addition of plasminogen (11) was adapted as follows. In 15 X 100 mm disposable petri plates the following materials were added: 10 ml of 7.5 mg/ml plasminogen free bovine fibrinogen (Miles Laboratories, Naperville, IL) in a 0.1 mol/l TRIS-HCl pH 8.0 buffer, 75 mg of human lys-plasminogen (American Diagnostica), and 4 IU plasminogen free

bovine thrombin (Miles Laboratories) in a 0.02 mol/l CaCl_2 + 0.15 mol/l NaCl solution. To the clots, 20 μl volumes of the conditioned medium sample at four serial two-fold dilutions were applied to each plate. After 18 hours at ambient temperature, the lysis zone diameters were read using a Transidyne General (Ann Arbor, MI) calibrating viewer (model 2743).

MICRO-CLOT LYSIS ASSAY (MCLA): This assay, developed in our laboratory was described by Lewis *et al.* (12) and utilizes 96 well flat bottom micro-well plates. The reaction mixture in the wells consisted of a fibrinogen solution containing plasminogen, thrombin, and the conditioned medium sample of PA standard. The optical density of the clots in each well was arbitrarily monitored at 405 nm at 5 minute intervals using an EIA automated microplate reader (Bio-Tek Instruments, Winoeski, VT, model EL310). The reader was connected via a serial interface to an Apple IIe computer. The computer received the data and determined the time at which the clots were half lysed, the assay endpoint, by use of software developed on the Apple IIe for this assay. As with the FPL assay, the activity for each sample was derived by comparison to the IRP-UK.

AMIDOLYTIC ASSAY (S-2444): The amidolytic assay using the synthetic substrate Pyro-Glu-Gly-Arg-pNA (S2444, KabiVitrum, Sweden) was essentially that described by Claeson *et al.* (13). For activation of latent PA in the conditioned medium, samples were incubated for 30 minutes at 37°C with human plasmin (American Diagnostica) at a final concentration of 2.3 $\mu\text{g/ml}$ of sample volume. Activation was stopped by the addition of aprotinin (Trasylol, FBA Pharmaceuticals, New York, NY) at 10 KIU/ml in a TRIS-NaCl-HCl buffer at pH 8.8. S-2444 was then added to this solution in

the wells of microtiter plates and the plates were incubated an additional 4 hours at 37°C. The absorbance was read at 405 nm using a Litton Bionetics (Charleston, SC) model LB130 microplate reader. The direct assay plates (without plasmin incubation) were handled in the same manner except that instead of addition of plasmin, an equivalent volume of the TRIS-NaCl-HCl buffer was added to samples.

TISSUE PLASMINOGEN ACTIVATOR (t-PA): The materials and methods for the t-PA assay were contained within the IMUBIND t-PA ELISA kit from American Diagnostica (14). The assay was modified using medium containing human serum albumen as a diluent. The flat bottom microwell plates (Immulon 2, Dynatech Laboratories, Alexandria, VA) plates were read at 490 nm on the EL310 autoreader.

PLASMIN INHIBITORS: To determine presence of plasmin inhibitors in the conditioned medium, samples were incubated with a known excess of human lys-plasmin. Residual plasmin activity was measured by the chromogenic substrate H-D-Val-Leu-Lys-pNA (S-2252, KabiVitrum, Sweden) at 405 nm (15) using the LB130 reader. Results are expressed as percent reduction in plasmin activity in the conditioned medium samples from the cultured cells compared to the unconditioned medium.

SODIUM DODECYL SULFATE POLYACRYLAMIDE ELECTROPHORESIS, (SDS PAGE), AND ZYMOGRAPHY: SDSPAGE electrophoresis to determine the molecular weight of the enzyme was performed as described by Laemmli (16). The resultant gels were analyzed for functional activity by development of a zymograph by the method of Tissot (17) involving a fibrin overlay.

ERYTHROPOIETIN: This assay was performed using a commercially available ELISA kit purchased from JCL Clinical Research Corp. (Knoxville, Tenn.). The analyses were performed as described in the methods brochure supplied with the kit.

HUMAN GRANULOCYTE COLONY STIMULATING ACTIVITY: This assay was performed using mouse marrow cells at a concentration of 75000 cells/ml. as described by Diersio et al (18).

RESULTS

Figure 1 shows the results on the conditioned media from the subpopulations by two assay for plasminogen activator activity, the fibrin plate lysis assay and the direct assay using the chromogenic substrate S-2444. As can be seen there is a large discrepancy between the activity levels as measured by these two different assays.

Figure 2 shows the results comparing the S-2444 direct with the S-2444 activated. These results show that much of the samples are in the single chain zymogen form and when activated with plasmin to the two chain form show a substantial increase in activity.

Figure 3A shows a comparison of the micro clot lysis assay with the fibrin plate lysis assay. As can be seen there is good correlation between the two assays except in the subpopulation region from fraction 9 thru 15. In Figure 3B these same fractions were analyzed for the presence of plasmin inhibitor and the fractions in the range 9-15 show significant

anti-plasmin activity. Thus the presence of anti plasmin in these fractions appear to be the cause for the discrepancy between the two different assays. The FPL assay appears to be independent of the presence of inhibitor while the MCLA assay is depended on the presence of such an inhibitor.

Figure 4 shows the entire set of data on the subfractions by the different assays. The results obtained are very dependent on the assay that is performed and points out dramatically the need to use a battery of assays to define the plasminogen activator profile of a sample from the conditioned media of cells in culture.

Figure 5A shows a typical fibrinogram on selected subpopulation samples. These fractions all show the presence of only species at 54,000 daltons, but do not distinguish between single and two chain forms. Figures 5B and 5C show the same gel on fibrinograms in which the fibrin overlay has been treated with specific antibody for u-PA to t-PA and allows one to determine the species present

While the majority of the analyses were to study the distribution of plasminogen activator(s), we did perform assays for two other biological activities associated with embryonic kidney cells in culture. The ELISA assay for erythropoietin showed areas of positive response but they were at a very low level. However, on triplicate determinations on the growth medium from run 4 there were consistent indications of a positive response in some subpopulations, these positive subpopulations showed levels of about 10 milli-immunological units per milliliter when compared with the standard. The assay kit as determined by our standard curve is insensitive to levels

below the 10 milliunits we detected. The mouse marrow assay for granulocyte colony stimulating activity was run on the growth media from both runs 3 and 4 and in both cases the results were negative.

DISCUSSION

The major conclusion that can be reached by this work is that a battery of assays are required to completely define the plasminogen activator profile of a conditioned media from cell culture. Each type of assay measures different parts of the mixture and are influenced by different parameters. Table 1 expresses the functional role of each assay and gives an indication of which combination of assays are required to answer specific questions. With this type of information it is possible by combinations of assays with mathematical analysis to pinpoint a specific component of the system. This problem can sometimes be simplified by first analyzing the samples by SDSPAGE and zymography with the specific antibodies to detect the different molecular forms and different molecular species present.

It was disappointing that the assays for human granulocyte colony stimulating activity were negative and that the assays for erythropoietin were at best marginal in the response that was obtained. Since ground based experiments have shown that embryonic kidney cells produce both of these biological activities when grown in culture, the lack of detectable activity in the space experiments must be due to the mechanics involved in the treatment of the cells and/or cultures.

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TABLE 1.

1. FIBRIN PLATE LYSIS

Diffusion based
Not affected by many inhibitors
Molecular weight dependent
Zymogen partially seen

2. MICRO CLOT LYSIS

Modification of classic tube lysis method
Measures uPA and tPA together all molecular weights
Zymogen partially seen
Inhibitors are functional

3. CHROMOGENIC = DIRECT ASSAY WITH S-2444

Sees both forms of uPA
Zymogen not seen
tPA most likely not seen
Only activator inhibitors measured

4. CHROMOGENIC = ACTIVATED ASSAY WITH S-2444

Sees all forms of uPA including zymogen
tPA not seen
Inhibitor same as S-2444

5. PLASMINOGEN ACTIVATION MEASURE PLASMIN BY CHROMOGENIC WITH S2251

Sees both forms UK
Zymogen partially seen
tPA not seen (modify by adding fibrin monomer)
Sees inhibitor

6. SDS PAGE WITH ZYMOGRAPHY

Shows all molecular weights for uPA-tPA
Qualitative, not quantitative
Does not differentiate zymogen

FIGURE 1.

Plasminogen activator activity in conditioned medium from human kidney (HK) cell subpopulations as detected by amidolytic S-2444 (○) and fibrin plate lysis (Δ) assays.

COMPARISON OF FIBRIN PLATE AND CHROMOGENIC ASSAYS

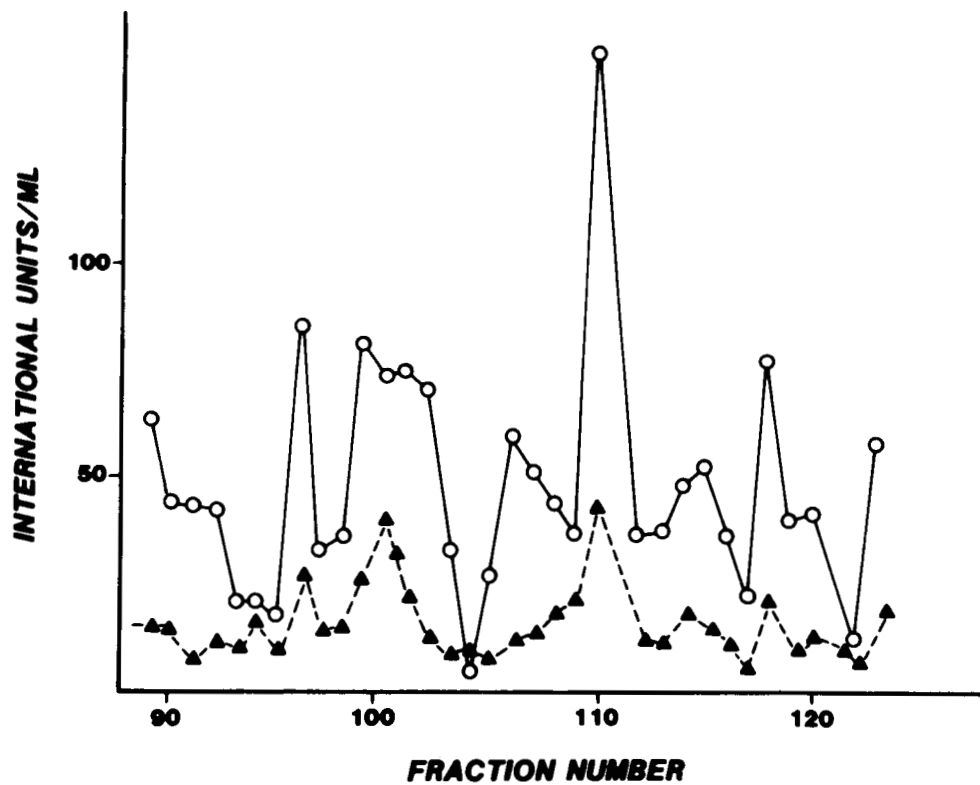


FIGURE 2.

Amidolytic activity detected after incubation of conditioned medium samples with plasmin for 30 minutes (activated, Δ) compared to activity of the same samples not incubated with plasmin (direct, \square).

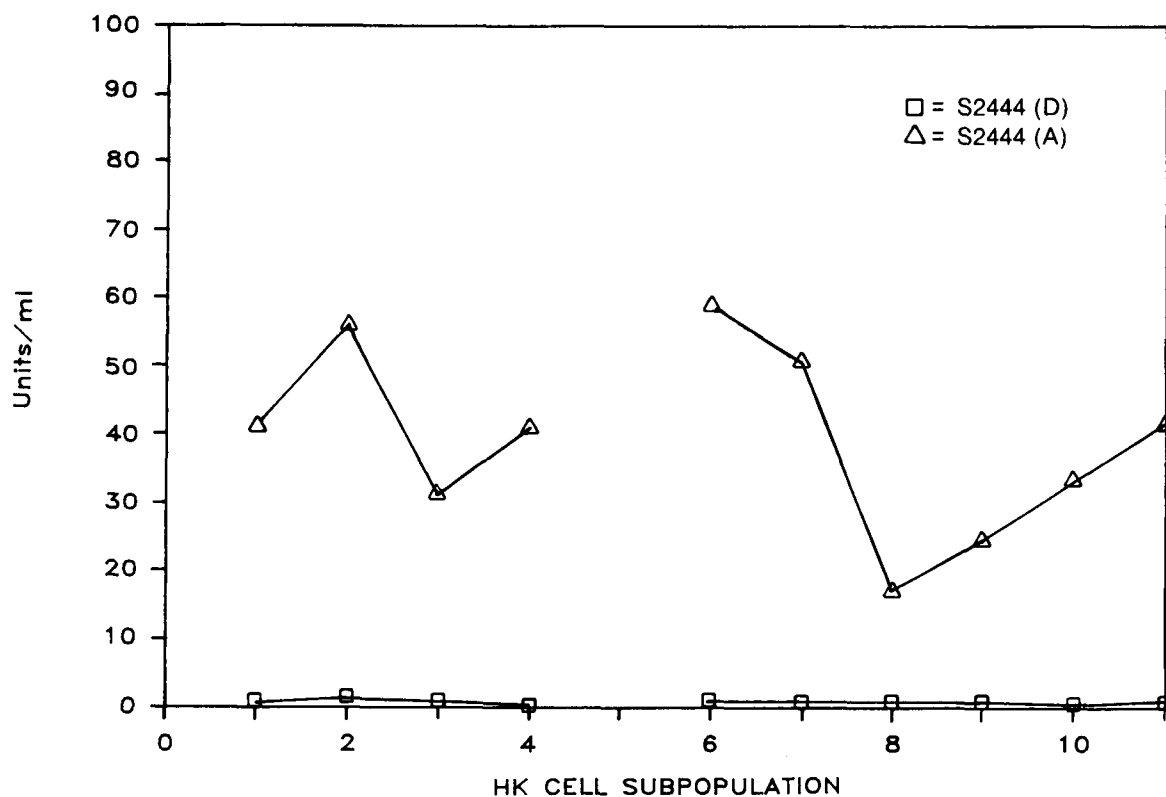


FIGURE 3A.

Comparison of the fibrin plate (+) and micro-clot lysis assays (□) for detecting fibrinolytic activity in conditioned medium from HK cell subpopulations. Both assays showed comparable activity except for the subpopulations in the range of 9-15.

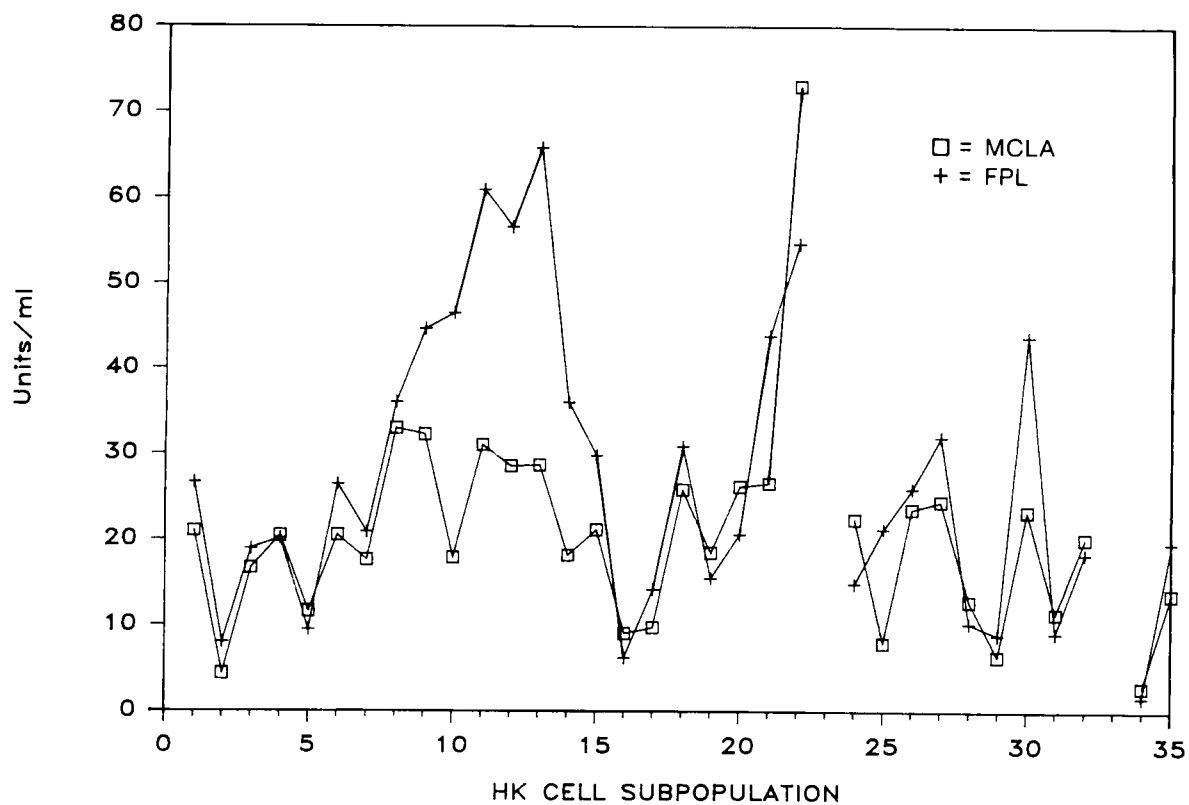


FIGURE 3B.

Presence of plasmin inhibitor in conditioned medium as detected by the chromogenic S-2251 (\square). Residual plasmin activity after addition of excess lys-plasmin to the samples is expressed as a percent of activity in unconditioned medium.

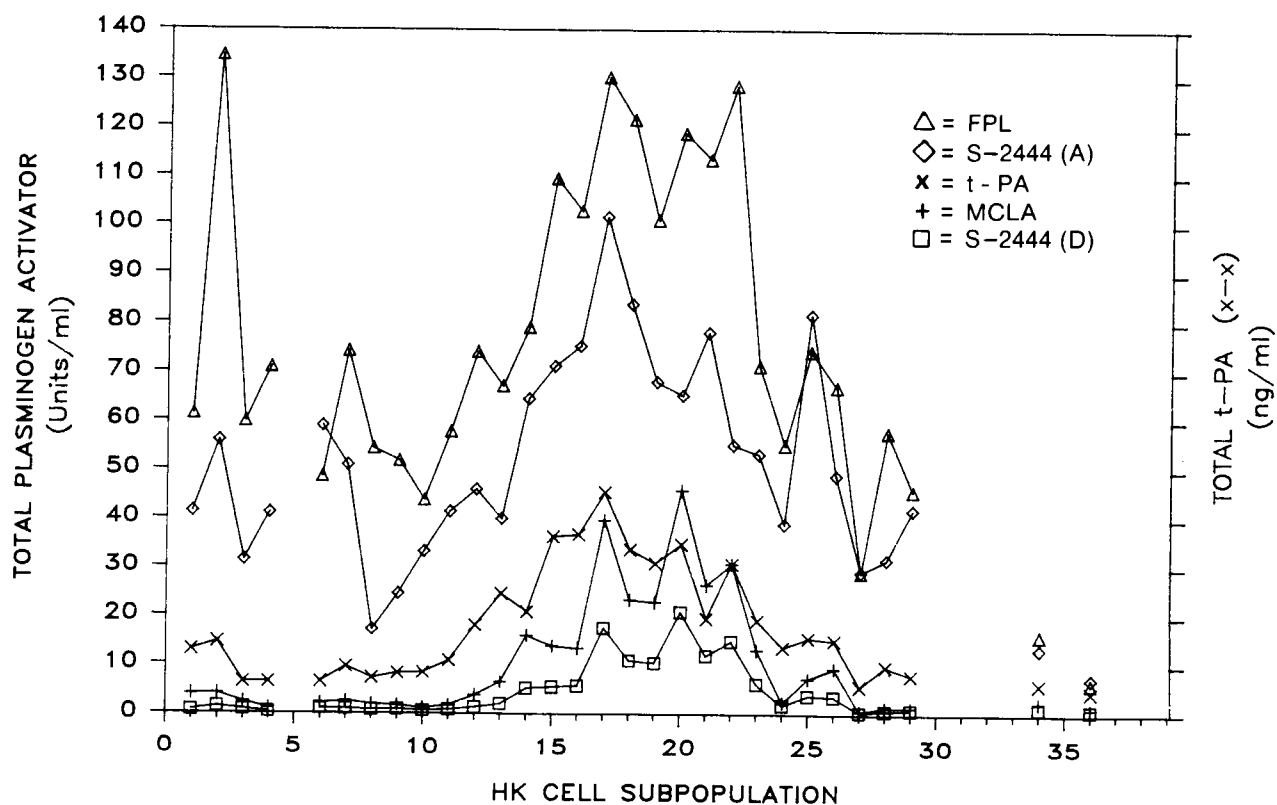
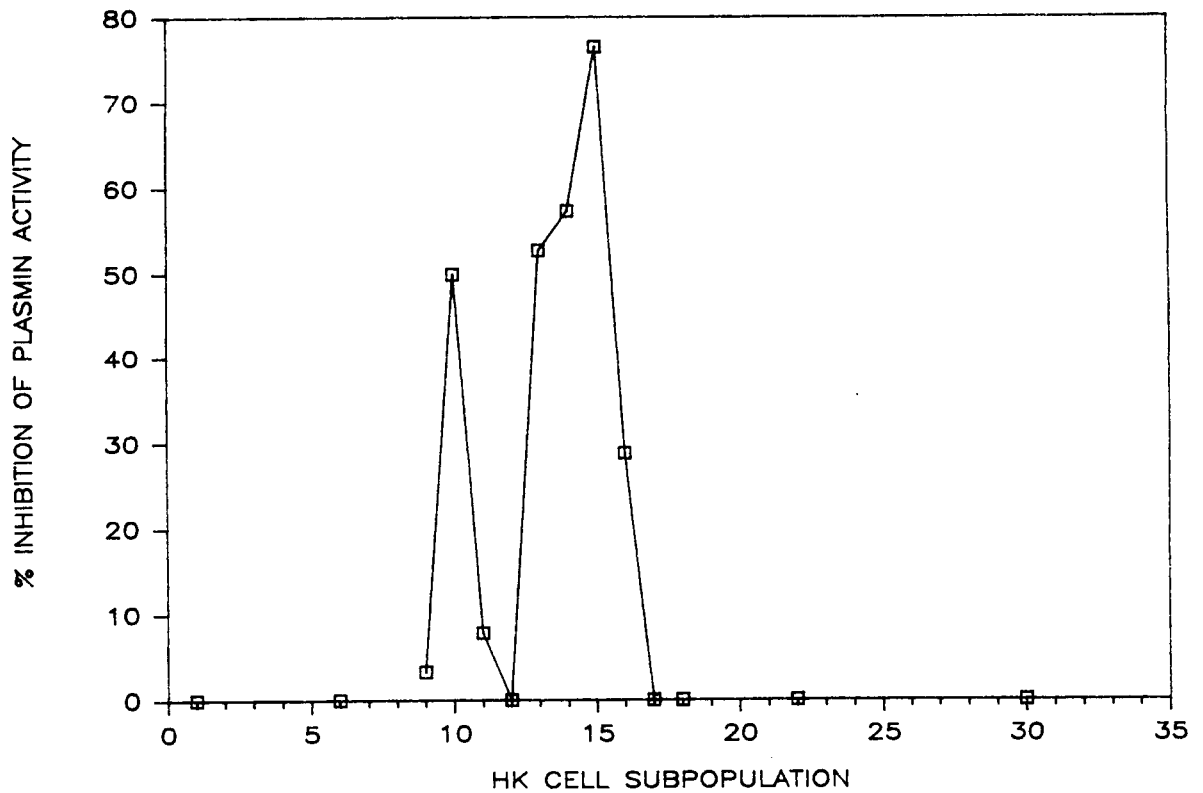


FIGURE 4.

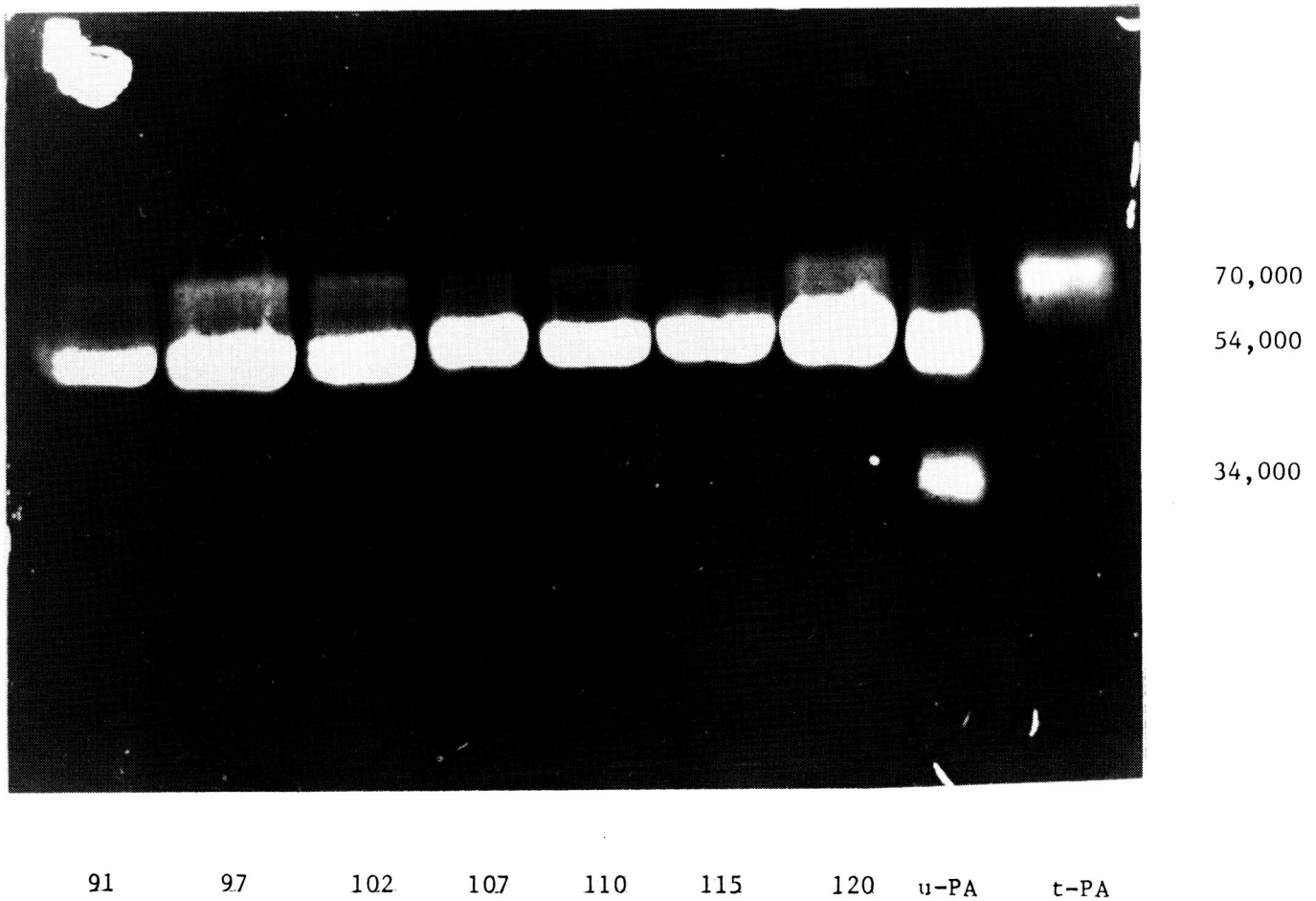
Plasminogen activators detected by a battery of five assays. These plots show the presence of active u-PA, (micro-clot lysis (+), S-2444 direct (□), and fibrin plate lysis (△) assays), scu-PA (difference between S2444 direct (□) and S-2444 activated (◇), and total t-PA in ng/ml enzyme linked immunosorbent assay) (x).



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FIGURE 5A.

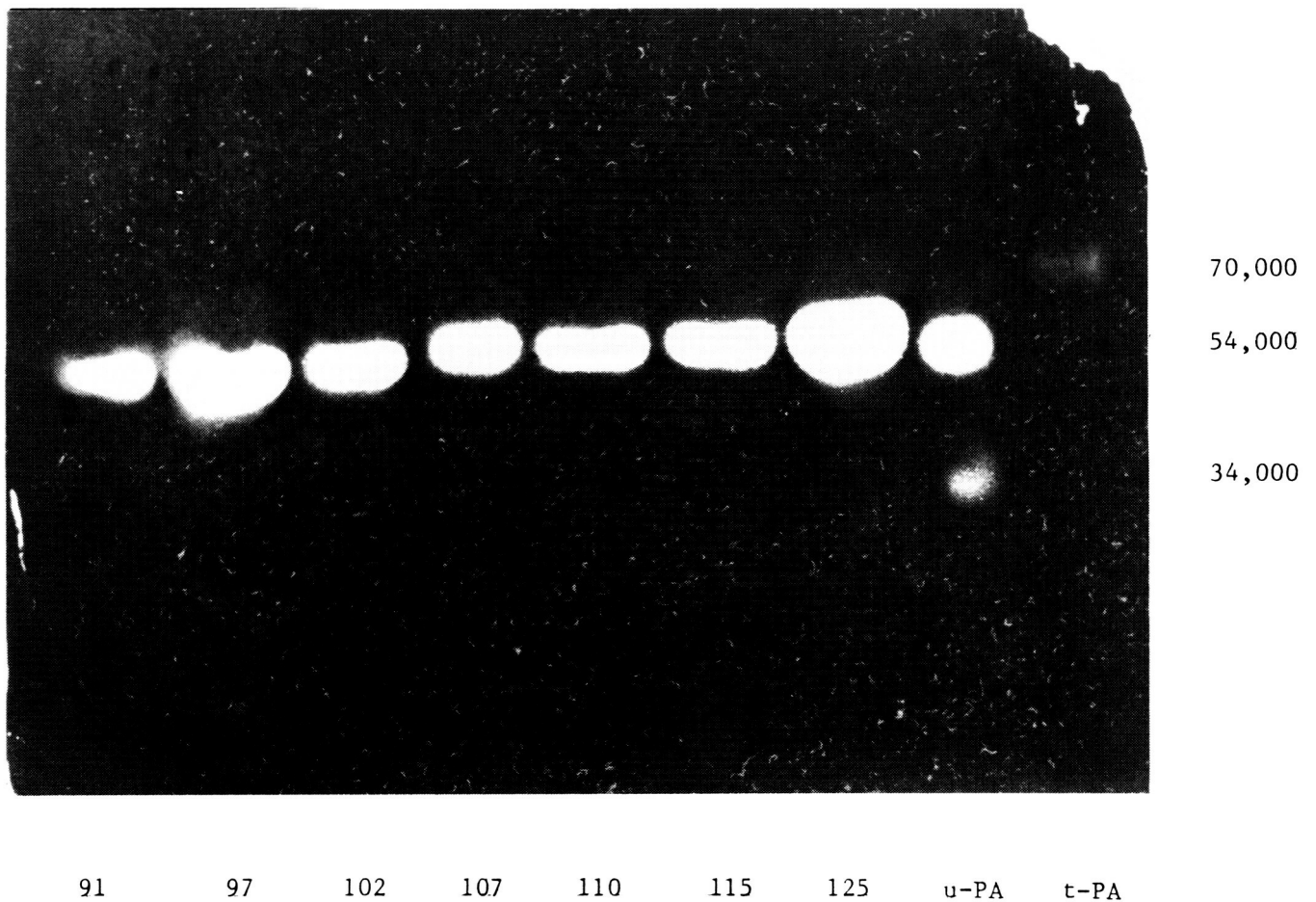
Fibrinogram from SDS PAGE gel on selected conditioned media samples from human embryonic kidney subpopulations.



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FIGURE 5B.

Fibrinogram from same SDS PAGE gel, overlay contains urokinase antibody.



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FIGURE 5C.

Fibrinogram from same SDS PAGE gel, overlay contains tissue plasminogen activator antibody.

